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Mode of Action of Naphthalic Anhydride as a Safener for the Herbicide AC 263222 in Maize

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Abstract: In hydroponic experiments, seed-dressing with the herbicide safener 1,8-naphthalic anhydride (NA), significantly enhanced the tolerance of maize, (*Zea mays* L., cv. Monarque) to the imidazolinone herbicide, AC 263222, (2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methylnicotinic acid). Uptake, distribution and metabolism studies where [^{14}C]AC 263222 was applied through the roots of hydroponically grown maize plants showed that NA treatment reduced the translocation of radiolabel from root to shoot tissue and accelerated the degradation of this herbicide to a hydroxylated metabolite. Reductions in the lipophilicity and, therefore, mobility of this compound following hydroxylation may account for NA-induced retention of radiolabel in the root system. Hydroxylation of AC 263222 suggested that NA may stimulate the activity of enzymes involved in oxidative herbicide metabolism, such as the cytochrome P_{450} mono-oxygenases. In agreement with this theory, the cytochrome P_{450} inhibitor, 1-aminobenzotriazole (ABT), synergized AC 263222 activity and inhibited its hyroxylation *in vivo*. NA seed-dressing enhanced the total cytochrome P_{450} and b_5 content of microsomes prepared from etiolated maize shoots. Isolated microsomes catalyzed AC 263222 hydroxylation *in vitro*. This activity possessed the characteristics of a cytochrome P_{450} mono-oxygenase, being NADPH-dependent and susceptible to inhibition by ABT. Activity was stimulated four-fold following NA seed treatment. Differential NA enhancement of AC 263222 hydroxylase and the cytochrome P_{450}-dependent cinnamic acid-4-hydroxylase (CA4H) activity, suggested that separate P_{450} isozymes were responsible for each activity. These results indicate that the protective effects of NA result from enhancement of AC 263222 hydroxylation and concomitant reduction in herbicide translocation. This may be attributed to the stimulation of a microsomal cytochrome P_{450} system. © 1998 SCI.

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Key words: naphthalic anhydride; AC 263222; herbicide metabolism; herbicide safener; cytochrome P_{450}; maize (*Zea mays*)

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1 INTRODUCTION

Herbicide safeners protect certain gramineous crop plants against herbicide damage by reducing the ability of herbicide molecules to reach and bind to their target sites in the crop plant. Theoretically, this may be achieved through safener-induced stimulation of herbicide target enzymes, reductions in herbicide uptake and translocation, or safener-enhanced metabolism of herbicides to less herbicidal or immobile metabolites. On investigation, safeners have been found to affect all of these processes, although evidence has now accumulated which advocates enhanced herbicide metabolism as the predominant mechanism of safener action.[1]

In particular, safeners have been demonstrated to stimulate the activity of glutathione transferase enzymes responsible for the conjugation of herbicides with glutathione during Phase II metabolism. However, safeners have also been shown to enhance crop tolerance to herbicides subject predominantly to the oxidative reactions of Phase I metabolism. These reactions are now widely accepted to be catalysed by cytochrome P_{450}-dependent mono-oxygenases associated with the smooth endoplasmic reticulum, i.e. microsomal fraction. However, until recently, the influence of safeners on such systems was unclear. While this may reflect difficulties in determining safener effects on *in-vivo* oxidative metabolism in cases where oxidation is followed by rapid conjugation with glucose or glutathione, safener induction of oxidative metabolism *in vitro* has also proved difficult to assess due to the low titre and extreme lability of these enzymes. Furthermore, safener enhancement of oxidative metabolism *in vivo* is not necessarily accompanied by increases in total cytochrome P_{450} content, possibly due to the induction of P_{450} isozymes specific for herbicide degradation at the expense of isozymes involved in the metabolism of endogenous substrates. However, since 1990, several workers have reported the enhanced ability of microsomes extracted from safener-treated monocotyledonous crop species to oxidize various herbicide substrates. For example, seed treatment with the safener naphthalic anhydride (NA) stimulated *in-vitro* hydroxylation of bentazone,[2] metolachlor,[2] primisulfuron,[2] nicosulfuron,[2] triasulfuron,[2–4] chlorsulfuron,[3,5] chlorimuron-ethyl,[6] diclofop-methyl,[3,5] chlorotoluron[5] and flumetsulam.[7] In all cases, activity was characteristic of cytochrome P_{450}, being NADPH-dependent and susceptible to inhibition by known cytochrome P_{450} inhibitors. In addition, the work of Baerg and Barrett (pers. comm.) suggests that NA also induces cytochrome P_{450} mono-oxygenases responsible for the metabolism of the imidazolinone herbicides imazethapyr and AC 263222.

In the present work, the activity of NA as a safener for the imidazolinone herbicide, AC 263222, in maize was investigated. AC 263222 is registered for the control of grass and broad-leaved weeds in peanuts but has been reported to cause injury to several following crops, including maize.[8] Consequently, the development of safeners to enhance maize tolerance to AC 263222 would not only extend the market for this herbicide into maize but also increase flexibility within rotational cropping systems. Furthermore, identification of the selectivity processes controlling maize tolerance through the study of safener mode of action may ultimately facilitate the development of AC 263222-resistant maize lines. This study was undertaken to identify the mechanisms responsible for NA activity, and evidence will be presented to suggest that this involves stimulation of a cytochrome P_{450} mono-oxygenase with the capacity for AC 263222 hydroxylation.

2 MATERIALS AND METHODS

2.1 Chemicals

Technical grade and [*pyridine*-^{14}C] AC 263222 (2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methylnicotinic acid) and its metabolite were gifts from American Cyanamid Company (Princeton, NJ, USA). Dr. I. Barta (Hungarian Academy of Sciences, Budapest, Hungary) supplied ABT (1-aminobenzotriazole) and MG 191 (2-dichloromethyl-2-methyl-1,3-dioxolane). NA (1,8-naphthalic anhydride, 98%) was obtained from Aldrich, Gillingham, Dorset, UK and *trans*-[3-^{14}C]cinnamate from Fluorochem Ltd., Old Glossop, Derbyshire, UK. All other reagents were purchased from Sigma Chemical Company, Poole, Dorset, UK

2.2 *In-vivo* effects of NA on AC 263222 activity, uptake, distribution and metabolism

Maize (*Zea mays* L; cv. Monarque) seeds were shaken with solid NA corresponding to 0·5% of their weight, reweighed and, if necessary, retreated until their target weight was achieved. Treated and untreated seed was then sown on damp filter paper in seed trays and germinated at 26°C and 80% RH in the dark. After two days, seedlings were suspended over plastic vials containing treatment solution (5 ml), such that seedling roots penetrated the solution to a depth of 1–2 cm. Alternatively, the shoots of three-day-old seedlings, excised close to the seed under water, were suspended such that they penetrated the solution by 0·5 cm. Treatment solutions contained AC 263222 or 16–20 kBq [^{14}C]AC 263222 (226 kBq μmol^{-1}) prepared in aqueous acetone (1 ml litre^{-1}) and half-strength Hewitt's nutrient medium.[9] Where required, this solution was supplemented with ABT. Vials were then returned to the growth cabinet and maintained with a 14-h photoperiod of light intensity 59 W m^{-2}.

In experiments monitoring whole-plant responses, seedlings were transferred to expanded polystyrene seed trays floating on 500 ml nutrient with continued ABT treatment as required. This was renewed at three-day intervals unit eight days after sowing (DAS) when roots and shoots were harvested for fresh weight determinations. Plants treated with [^{14}C]AC 263222 were harvested 3, 4 or 7 DAS, while excised shoots were harvested 4 DAS. At all stages, used nutrient solutions were retained for liquid scintillation counting.

Tissue samples were extracted in acetone + methanol + water (1 + 1 + 1 by volume; 10 ml per g tissue), using an Ultra-Turrax homogenizer for 3 × 1 min. The homogenate was then centrifuged at 13 000 rev min^{-1} (Microcentrifuge, MSE) for 10 min, the supernatant decanted and the pellet resuspended. Following a second extraction, the pellet was combusted using a Harvey Biological Oxidizer OX500 and the resulting [^{14}C] carbon dioxide trapped in Carbomax scintillant (15 ml) and counted for 2 min in a LKB RackBeta II counter. Supernatants were combined and filtered through Whatman No 1 filter paper before being reduced to dryness under reduced pressure at 50°C. Flasks were then rinsed with extraction solvent (2 × 2 ml) before evaporation to dryness under a stream of nitrogen at 40°C. Residues were resuspended in 200 μl solvent, 100 μl of which was mixed with 3·0 ml scintillant (OptiPhase HiSafe 3) and counted as before. The remaining extract was spotted onto TLC plates (Merck, Kieselgel 60 F_{254}) for development in *n*-propanol + dichloromethane + formic acid (4 + 5 + 1 by volume). After drying, parent AC 263222 and its metabolites were located using an Isomess Plate Scanner. Areas of silica gel corresponding to ^{14}C peaks were then scraped into vials, eluted with methanol (500 μl) for 1 h and subjected to liquid scintillation counting as before. Metabolites were identified by co-chromatography with technical grade standards.

2.3 Microsome preparation

Seeds of maize, cv. Pioneer 3343IR, dressed with 0·5% (w/w) NA where necessary, were sown between layers of damp germination paper and maintained under darkness at 27°C. After 3·5 days, etiolated shoots were harvested and combined with extraction buffer (1 ml g^{-1}) composed of sodium phosphate, (0·1 M; pH 7·4), sucrose (250 mM) ascorbic acid (40 mM) mercaptoethanol, (25 mM) and EDTA (1 mM). Tissue was ground for 30–60 s using a Polytron homogenizer and filtered through two layers of Miracloth prior to centrifugation at 15 000*g* and 4°C for 20 min. The resulting supernatant was then decanted and centrifuged at 100 000*g* for 1 h to sediment the microsomal pellet. This was resuspended in sodium phosphate, (0·1 M; pH 7·5) with mercaptoethanol (10 mM) and EDTA (1 mM), before recentrifugation at 100 000*g* for 1 h. The final pellet was resuspended in sodium phosphate (0·1 M; pH 7·4) with mercaptoethanol (1·5 mM) and glycerol (300 g kg^{-1}) and stored at −80°C until required. Microsomal protein contents were estimated using the Pierce Coomassie protein assay system.

2.4 Measurement of microsomal enzyme activities

2.4.1 Cytochrome b_5 and cytochrome P_{450} contents

Cytochrome b_5 and cytochrome P_{450} contents of microsomal extracts were estimated according to the methods of Omura and Sato.[10] Microsomes were diluted with sodium phosphate, (0·1 M; pH 7·4), to a protein concentration of 0·5–1·0 mg ml^{-1}. A volume of 1·0 ml was transferred to a glass cuvette and a base-line spectrum generated between 400 and 600 nm using a Hewlett Packard 8452A diode-array spectrophotometer. The sample was then reduced for 1 min with sodium dithionite before scanning the reduced minus air-oxidized difference spectrum. This was used to estimate cytochrome b_5 content assuming an extinction coefficient of 185 mM^{-1} cm^{-1} for $A_{409-424\,nm}$.

A new base-line was generated before the sample was bubbled with carbon monoxide (CO) for 45 s. A final scan produced the CO-treated minus reduced difference spectrum from which cytochrome P_{450} content was calculated using an extinction coefficient of 91 mM^{-1} cm^{-1} for $A_{450-490\,nm}$.

2.4.2 Cinnamic acid-4-hydroxylase

Using the methods of Reichart *et al.*,[11] cinnamic acid-4-hydroxylase (CA4H) activity was assayed with microsomal protein (0·5–1·0 mg) and NADPH (0·4 μmol), in a total volume of 200 μl sodium phosphate buffer (0·1 M; pH 7·4). The reaction was initiated by the addition of a 20-μl aliquot of a substrate mixture prepared in ethanol phosphate buffer (10 + 90 by volume) containing *trans*-[3-^{14}C]cinnamate (0·4 nmol; 2098 kBq μmol^{-1}) and *trans*-cinnamate (200 nmol). Reaction mixtures were transferred to a shaking water bath operating at 28°C. After 20 min incubation, the reaction was terminated by the addition of hydrochloric acid (4 M; 20 μl) followed by 10 μl ethanol containing 0·7 μmoles each of *trans*-cinnamic and *p*-coumaric acids. Samples were then centrifuged for 5 min at 13 000 rev min^{-1} (Microcentrifuge, MSE) to remove precipitated protein before 100 μl supernatant was spotted in a 2·5 cm line on fluorescent silica gel TLC plates (Merck, Kieselgel 60 F_{254}).

Plates were developed in toluene + acetic acid + water (6 + 7 + 3 by volume, upper phase). The substrate, *trans*-cinnamic acid, and the metabolite, *p*-coumaric acid, were identified by co-chromatography with technical grade compounds which fluoresce under UV light at R_f 0·47 and 0·24, respectively.

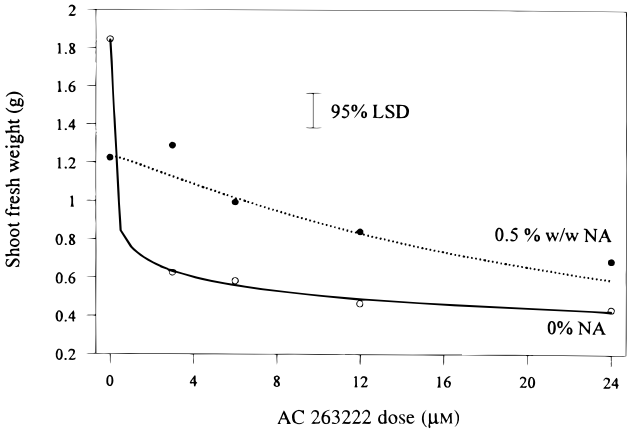

Fig. 1. Effect of napthalic anhydride (NA) on the activity of AC 263222 in hydroponic maize cultures. Two-day-old maize seedlings grown from undressed and NA-dressed seed were treated with AC 263222 applied in 0·5% aqueous acetone through nutrient solution for 24 h. Plants were then transferred to untreated nutrient and maintained for six days before harvest.

2.4.3 AC 263222 hydroxylase

In-vitro metabolism of AC 263222 was assayed in a final volume of 200 μl sodium phosphate buffer (0·1 M; pH 7·4) with 0·5–2·0 mg microsomal protein and a 20-μl aliquot substrate mixture containing [^{14}C]AC 263222 (20 nmol; 226 kBq μmol^{-1}) prepared in acetone + phosphate buffer (4 + 96 by volume). The reaction was initiated by addition of a 20-μl aliquot of NADPH (0·4 nmol) in phosphate buffer. In separate experiments, the reaction mixture was supplemented with ABT (250 or 500 μM) prepared in phosphate buffer and added as a 20-μl aliquot. Samples were incubated at 30°C for 2 h when the reaction was terminated by addition of acetic acid (25 μl). Parent AC 263222 and its metabolite were extracted with ethyl acetate (3 × 1 ml) and reduced to dryness under air at room temperature. The resulting residue was taken up in acetone + methanol + water (1 + 1 + 1 by volume; 200 μl) and analysed by TLC as described for *in-vivo* AC 263222 metabolism.

In cinnamate-4-hydroxylase and AC 263222 hydroxylase assays, control samples were included in which NADPH and/or microsomes were replaced with buffer. Activities were calculated by subtraction of background levels of metabolite production seen in microsome samples incubated without NADPH.

2.5 Experimental design and statistical analysis

The effects of NA and ABT on AC 263222 activity were investigated in factorial experiments incorporating six herbicide doses applied with and without NA or ABT with five replicate plants per treatment. Experiments investigating the effects of safeners on selectivity processes incorporated four replicates of two plants for root extractions and two replicates of four plants for shoot extractions. All data were subject to ANOVA using GENSTAT 5.0 and significant herbicide dose responses were described by a logistic curve. The effects of safener and synergist treatments on the GID_{50} parameter (dose causing 50% inhibition of growth) of this curve were determined as described by Davies.[12]

3 RESULTS

3.1 Effect of NA on AC 263222 activity

Seed treatment with 0·5% (w/w) NA caused significant reductions in the root and shoot fresh weights of hydroponically grown maize seedlings. For example, shoot

TABLE 1

Effects of Napthalic Anhydride (NA) and 1-Aminobenzotriazole (ABT) on the Uptake of [^{14}C]AC 263222 by Hydroponically Grown Maize Seedlings. Two-day-old maize seedlings grown from NA-dressed and undressed seed were treated with [^{14}C]AC 263222 with or without ABT *via* nutrient solution for 24 h. Seedlings were then harvested at 3 DAS. Alternatively, shoots of three-day-old seedlings were excised and treated with AC 263222 until harvest 4 DAS

Tissue	*Treatment*	*Dose* (% *w/w*)	*Uptake* (*dpm mg*$^{-1}$ *fresh weight*)	*Change following treatment* (%)	*95% LSC*[a]
Chlorophyllous seedlings	NA	0	205·2	−18·9	14·2
		0·5	166·5		
Achlorophyllous seedlings	NA	0	205·8	−30·4	14·2
		0·5	143·3		
Chlorophyllous excised shoots	NA	0	720·5	−10·4	14·2
		0·5	652·0		
Chlorophyllous seedlings	ABT	0 μM	76·3	10·5	16·2
		40	68·3		

[a] Least significant change.

TABLE 2
Effect of Napthalic Anhydride (NA) on the Distribution of AC 263222 between Maize Root and Shoot Tissue. Two-day-old maize seedlings grown from NA-dressed and undressed seed were treated with [^{14}C]AC 263222 applied *via* nutrient solution for 24 h. Seedlings were then transferred to untreated nutrient which was replaced at two-day intervals until harvest at 3, 4 or 7 days

	Radiolabel in shoots (% of total absorbed)			
	Seedling age (days)			
NA dose (% w/w)	*3*	*4*	*7*	*95% LSD*[a]
0	2·9	13·9	30·0	3·9
0·5	2·8	6·5	8·6	

[a] Least significant difference.

fresh weight was reduced from 1·85 g to 1·24 g following NA treatment. Nevertheless, NA significantly enhanced the tolerance of maize seedlings to root-applied AC 263222, increasing the GID_{50} estimated from shoot fresh weight data, from 0·241 to 22·2 μM (Fig. 1). Similarly, the GID_{50} estimated from root fresh weight measurements was increased from 0·049 to 11·5 μM.

3.2 Effect of NA on AC 263222 uptake, distribution and metabolism *in vivo*

Seed dressing with 0·5% (w/w) NA significantly reduced root uptake of AC 263222 by chlorophyllous maize seedlings, from 205 to 167 dpm mg^{-1} (Table 1). Once in the root system, radiolabel was either translocated to the shoot or exuded into the nutrient. Treatment with NA significantly reduced these processes, such that a greater proportion of radiolabel was retained in the root system. For example, although shoots of treated and untreated seedlings contained similar amounts of radiolabel at 3 DAS, only 9% of total absorbed radiolabel had been translocated to the shoots of safened plants by day 7, while 30% had been translocated in unsafened plants (Table 2). Furthermore, while untreated seedlings had exuded 35% of total radiolabel back into the nutrient by day 7, safened plants had exuded only 21%.

More than 97% of total absorbed radiolabel was extracted from maize tissue irrespective of NA treatment. This indicates that significant levels of AC 263222 were not metabolized to insoluble, bound residues and that such metabolism was not induced by NA. Meanwhile, TLC analysis of maize extracts indicated that AC 263222 was degraded to a single metabolite which was identified by co-chromatography as AC 263222 hydroxylated on the methyl group of the pyridine ring. Production of this metabolite by the root tissue of chlorophyllous seedlings was doubled from 109 to 217 dpm mg^{-1} following NA seed treatment. Similar responses were observed in shoot tissue. However, the true relative metabolic capacity of NA-treated and untreated seedlings may be obscured by low concentrations of [^{14}C]AC 263222 reaching the shoot system due to retention of radiolabel in the root system of NA-treated seedlings. Consequently, the effects of NA on shoot metabolism were evaluated by pulsing [^{14}C]AC 263222 directly into shoots excised from NA-treated

TABLE 3
Effects of Naphthalic Anhydride (NA) and 1-Aminobenzotriazole (ABT) on the Hydroxylation of AC 263222 by Hydroponically Grown Maize Seedlings. Two-day-old maize seedings grown from NA-dressed and undressed seed were treated with [^{14}C]AC 263222 with or without ABT *via* nutrient solution for 24 h. Seedlings were then harvested at 3 DAS. Alternatively, shoots of three-day-old seedlings were excised and treated with AC 263222 until harvest 4 DAS

Tissue	*Treatment*	*Dose (% w/w)*	*Hydroxylated AC 263222 (dpm mg^{-1} fresh weight)*	*Change following treatment (%)*	*95% LSC*[a]
Chlorophyllous seedling root	NA	0	109·5	98·2	19·5
		0·5	217·0		
Achlorophyllous seedling root	NA	0	131·9	67·0	19·5
		0·5	220·1		
Chlorophyllous excised shoots	NA	0	137·0	285·7	96·2
		0·5	528·5		
Chlorophyllous seedling roots	ABT	0 μM	93·2	−47·2	22·2
		40	49·2		

[a] Least significant change.

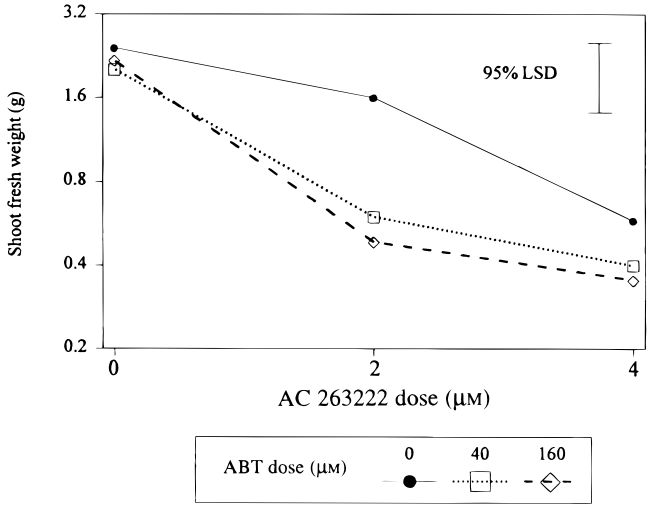

Fig. 2. Effect of 1-aminobenzotriazole (ABT) on the activity of AC 263222 in hydroponic maize. Two-day-old maize seedlings were treated with ABT and AC 263222 applied in 0·5% aqueous acetone through nutrient solution for 24 h. Plants were then transferred to fresh nutrient with continued ABT treatment until harvest 6 DAS.

seedlings. Using this system, NA pre-treatment did not significantly affect the uptake of AC 263222 but increased AC 263222 hydroxylation almost four-fold, from 137 to 528 dpm mg^{-1} (Table 3).

The interference of plant pigments in the measurement of microsomal enzyme activities necessitates the use of achlorophyllous, i.e. etiolated plant material. In anticipation of this problem, the effects of NA on AC 263222 uptake, distribution and metabolism were also investigated in three-day-old etiolated seedlings. This revealed that seed-dressing with 0·5% NA significantly reduced uptake from 206 to 143 dpm mg^{-1} (Table 1) but did not alter the distribution of radiolabel between root and shoot tissue. In contrast, NA significantly enhanced metabolite production from 132 to 220 dpm mg^{-1} in root tissue. These effects were not significantly different from those observed in three-day-old chlorophyllous seedlings, thus indicating that NA activity is independent of light and validating the use of etiolated material for *in-vitro* experiments.

3.3 Effect of ABT on AC 263222 activity, uptake, distribution and metabolism *in vivo*

The hydroxylated nature of the AC 263222 metabolite suggested that metabolism may be catalyzed by a cytochrome P_{450}-dependent mono-oxygenase. In agreement with this theory, root applications of the cytochrome P_{450} inhibitor, aminobenzotriazole (ABT) were found to synergize AC 263222 activity in Monarque seedlings. Although not inhibitory when applied alone, ABT root treatments significantly increased AC 263222 activity. Maize shoot fresh weight was reduced from 2·4 g to

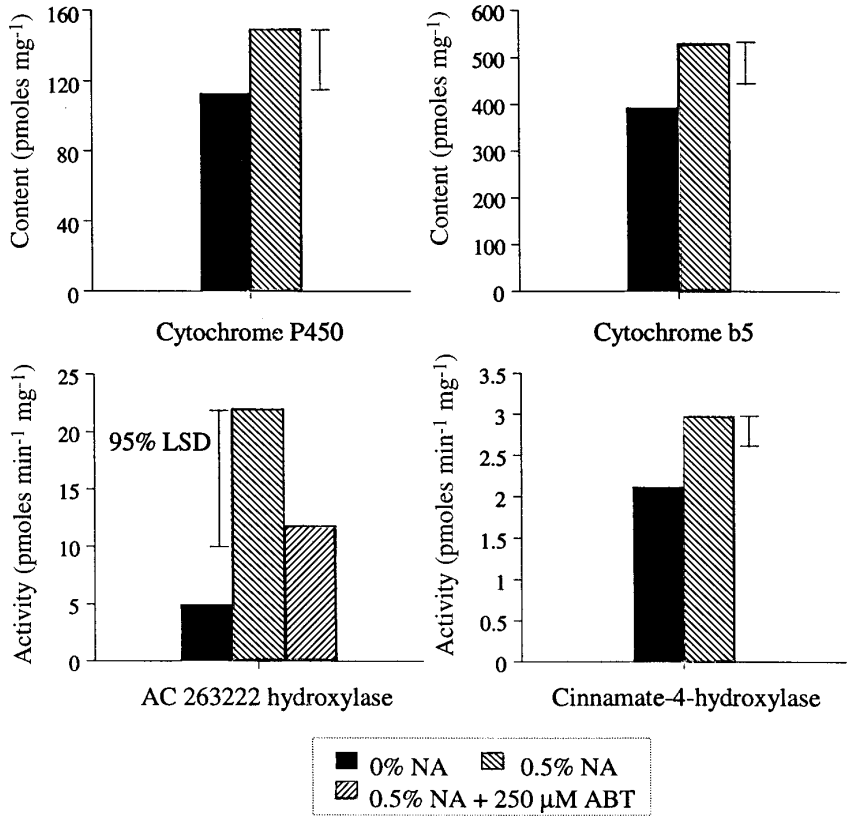

Fig. 3. Effect of naphthalic anhydride (NA) and 1-aminobenzotriazole (ABT) on cytochrome P450-related activities of microsomes extracted from etiolated maize shoots. NA treated and untreated seed was germinated in the dark for 3·5 days. Shoot tissue was then excised and microsomes prepared and activities assayed as described in the text. ABT treatments were added at incubation.

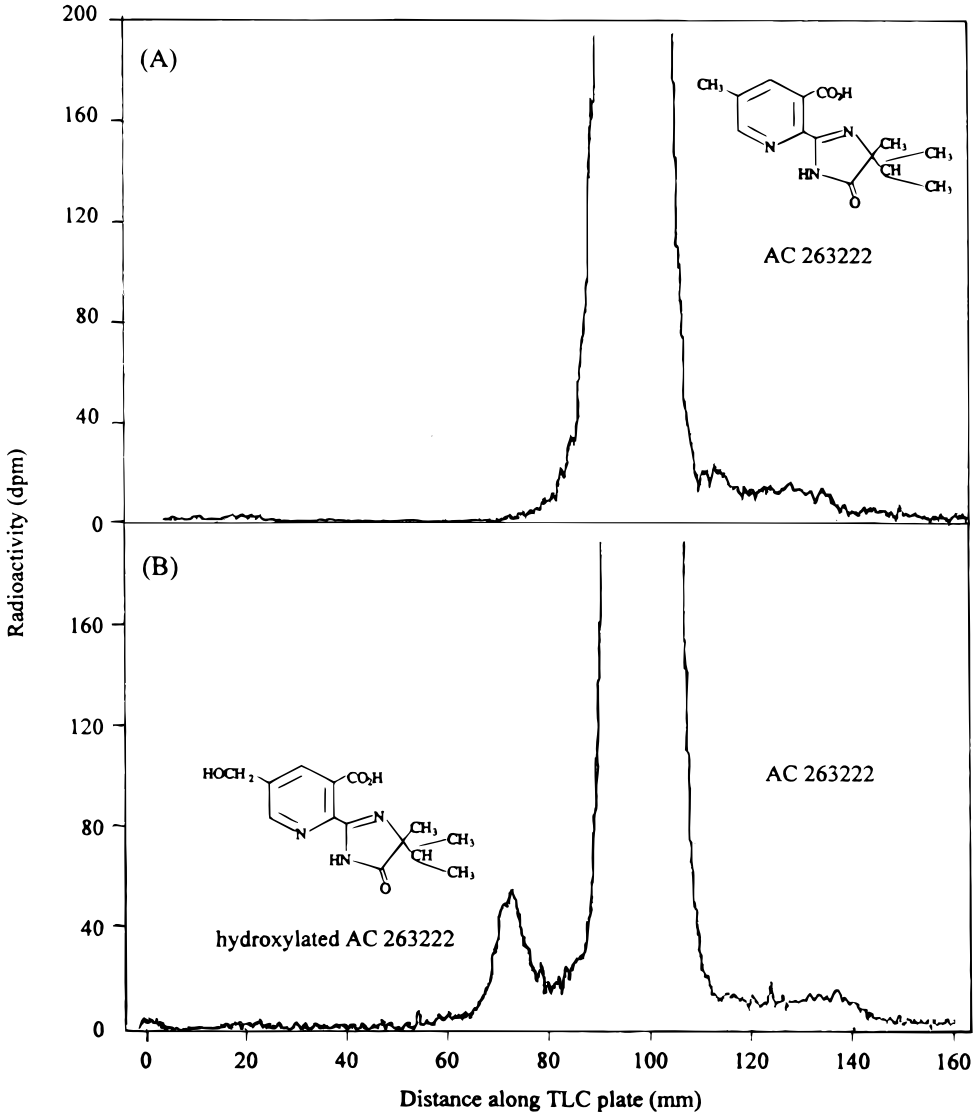

Fig. 4. Radiochromatographs showing NADPH-dependent AC 263222 hydroxylation by microsomes extracted from 3·5-day-old shoots of the maize variety Pioneer 3343IR. (A) Microsomes incubated alone. (B) Microsomes incubated with NADPH. TLC solvent = propanol + dichloromethane + formic acid (4 + 5 + 1 by volume).

1·6 g with 2 μM AC 263222, but to 0·6 g when synergized with 40 μM ABT (Fig. 2). This synergistic effect was not significantly enhanced by increases in ABT dose to 160 μM. Root applications of 40 μM ABT did not significantly alter the uptake of [^{14}C]AC 263222 (Table 1), its distribution between root and shoot tissue or its extractability from maize tissue. However, hydroxylation of AC 263222 by root tissue was approximately halved following ABT treatment (Table 3).

3.4 Effect of NA and ABT on cytochrome P_{450}-dependent activities *in vitro*

Further evidence for the interaction of NA with cytochrome P_{450} systems was obtained from *in-vitro* studies with microsomes extracted from 3·5-day-old etiolated Pioneer 3343IR shoots. In agreement with spectra obtained by Vanden Bossche *et al.*,[13] the CO minus reduced difference spectrum of maize shoot microsomes was characterized by a major peak at 450 nm and a shoulder at 420 nm. Total cytochrome P_{450} content was significantly increased from 113 to 150 pmol min^{-1} mg^{-1} protein following seed treatment with 0·5% NA, while cytochrome b_5 content was promoted from 393 to 532 pmol min^{-1} mg^{-1} (Fig. 3). Cinnamate-4-hydroxylase activity was identified by the appearance of *p*-coumaric acid at R_f 0·24 in microsome samples incubated with NADPH and was 40% greater in microsomes extracted from NA-treated seedlings than in those extracted from untreated seedlings, (Fig. 3).

Hydroxylation of AC 263222 was also performed by Pioneer 3343IR microsomes incubated with NADPH at 30°C for 2 h. Under these conditions, activity was detected by the appearance of a metabolite at R_f 0·45

which co-chromatographed with technical grade AC 263222 hydroxylated at the methyl group of the pyridine ring (Fig. 4). Hydroxylated AC 263222 was not detected in control samples incubated without NADPH and/or microsomes. This indicates that metabolism was enzymatic and NADPH-dependent. Seed treatment with 0·5% (w/w) NA significantly promoted microsomal AC 263222 hydroxylase activity from 5 to 22 pmol min^{-1} mg^{-1} protein. Incubation of microsomes extracted from NA-treated seedlings with 250 μM ABT reduced the rate of AC 263222 hydroxylation from 22·1 to 11·8 pmol min^{-1} mg^{-1} protein (Fig. 3). This level of inhibition was not improved by increases in ABT concentration to 500 μM.

4 DISCUSSION

4.1 Reduced herbicide uptake as a mode of safener action

Results presented herein suggest that inhibition of AC 263222 uptake may contribute to the protective effect of NA. This possibility was investigated further by Davies *et al.*,[14] who reported similar reductions in uptake following treatment with the safener, MG 191, which did not have significant protective activity. This suggests that reductions of the magnitude observed do not reduce internal herbicide concentrations to non-phytotoxic levels and, thus, inhibition of uptake cannot be the predominant mechanism of safener action. The importance of reduced uptake was further undermined by the observation that protective effects can be achieved with NA root treatments applied 24 h after AC 263222. Under these circumstances, opportunities for NA interference with uptake processes have been eliminated. However, as activity was significantly lower than that induced by NA applied with or after AC 263222, the possibility that reduced uptake may contribute to safener activity cannot be eliminated. Other investigations of safener effects on uptake processes have produced a series of contradictory results. NA has been reported to increase herbicide uptake,[15–17] have no effect[18,19] and reduce herbicide uptake.[20,21] Consequently, little importance is attached to these effects and, where changes have been observed, their origin remains largely unquestioned.

Ketcherisid *et al.*[22] proposed that cyometrinil-induced decreases in metolachlor uptake may result from reductions in membrane permeability. Contrary to this theory, permeability, as estimated by the exudation of ^{14}C-photosynthate from sorghum roots, was significantly enhanced by cyometrinil treatment. However, in our experiments, NA seed dressings significantly reduced exudation and translocation of radiolabel, thus suggesting that NA may influence membrane permeability. Indeed, the continued accumulation of weakly acidic molecules, such as the imidazolinones, against a concentration gradient requires the activity of proton-ATPases responsible for the maintenance of pH gradients across membranes. Consequently, NA interference with respiratory processes may cause reductions in imidazolinone uptake. Although NA is not reported to induce such effects, it is known to inhibit maize germination and growth.[12] Other safeners, such as the oxime ethers, have been demonstrated to inhibit the respiration of germinating sorghum seedlings[23] and interfere with numerous metabolic processes including photosynthesis, protein, nucleic acid and lipid synthesis.[24]

Alternatively, given that root uptake of the imidazolinones is proportional to transpiration rates, inhibition of transpiration processes by safener treatments may lead to reductions in root uptake. However, the failure of NA to inhibit uptake of radiolabel by excised shoots where uptake occurs by capillary action and is therefore dependent on transpiration rates suggests that NA has no direct effect on transpiration. Furthermore, it suggests that the mechanisms responsible for NA inhibition of herbicide uptake are associated with the root system.

4.2 Enhanced metabolism and reduced translocation as a mode of safener action

NA clearly stimulated hydroxylation of AC 263222 by maize root and shoot tissue. As the hydroxylated metabolite is also a potent ALS inhibitor (Shaner, pers. comm.), enhanced metabolism alone cannot account for the ability of NA to reduce herbicide injury. However, the increased polarity and, therefore, reduced lipophilicity of AC 263222 following hydroxylation, would limit its ability to penetrate membranes and its mobility relative to that of the parent molecule.[25] In agreement with this suggestion, NA was found to impede translocation and root exudation processes, leading to increased retention of radiolabel in the root system. This hypothesis also implies that parent AC 263222 would be preferentially exuded from the roots, although this was not confirmed experimentally. These findings closely follow those of Barrett and Maxson[26] and Shaner[27] who made similar observations when investigating the interaction between NA and the closely related imidazolinone, imazethapyr.

Synergism of AC 263222 activity and *in-vivo* inhibition of herbicide hydroxylation by the cytochrome P_{450} inhibitor ABT, suggests that AC 263222 metabolism may be catalyzed by a cytochrome P_{450} monooxygenase. Similar observations were made by Shaner[27] who used ABT inhibition of oxidative herbicide metabolism to implicate cytochrome P_{450} in the hydroxylation of imazethapyr. Although Reichhart *et al.*[28] present evidence for the specificity of ABT as a suicide

substrate for cytochrome P_{450}, this inhibitor has been found to influence other biochemical and physiological processes. For example, Blee and Durst[29] observed inhibition of sulphoxidase and chloroperoxidase activities in maize microsomes, while Sterling and Balke[30] reported inhibition of bentazone uptake by soybean cell suspension cultures. Consequently, this observation does not provide conclusive evidence for the catalysis of AC 263222 hydroxylation by cytochrome P_{450}.

In contrast, substantial evidence was provided by *in-vitro* studies which demonstrated the ability of microsomes prepared from etiolated maize shoots to perform AC 263222 hydroxylation. This activity was characteristic of a cytochrome P_{450} mono-oxygenase being NADPH-dependent and susceptible to inhibition by ABT and stimulation by NA. Indeed, the effects of ABT and NA on AC 263222 hydroxylase activity *in vivo* and *in vitro* are extremely well correlated. For example, both *in-vivo* and *in-vitro* studies revealed approximately four-fold increase in AC 263222 hydroxylation following NA treatment, and a 50% reduction with ABT treatment. These observations provide strong evidence for cytochrome P_{450} catalysis of AC 263222 hydroxylation and suggest that the protective effects of NA result from stimulation of this system. This mode of action is also advocated by observation of elevated cytochrome P_{450} content and cinnamate-4-hydroxylation in NA-treated tissue. Furthermore, NA-induced increases in cytochrome, b_5, a component of the microsomal electron transfer reactions of cytochrome P_{450}, could also contribute to a rise in the metabolic capacity of a cytochrome P_{450}-based herbicide hydroxylating system.

Results presented here also suggest that AC 263222 hydroxylation is performed by a minor, yet specific P_{450} isozyme. In particular, differential enhancement of cinnamate-4-hydroxylase and AC 263222 hydroxylase by NA suggests that distinct isozymes are responsible for each activity. Furthermore, the greater magnitude of the NA-induced increase in AC 263222 hydroxylase compared with the increase in total cytochrome P_{450} content also advocates the specificity of AC 263222 hydroxylase. Similar suggestions were made by Salaün *et al.*[31] following observation of differential induction of lauric acid hydroxylase and total cytochrome P_{450} content by clofibrate and 2,4-D in Jerusalem artichoke. These authors proposed that physiological regulatory mechanisms may offset the promotion of a specific isozyme by suppression of others, thus, leading to minor changes in overall content.

In the future, characterization of NA-inducible isozymes and definition of their substrate specificity may explain the ability of NA to provide protection against a wide range of herbicidal molecules and also enable prediction of antagonistic herbicide-safener interactions. Furthermore, the identification of genes encoding NA-inducible enzymes may ultimately facilitate the development of imidazolinone-resistant crop lines with an enhanced capacity for herbicide metabolism. This approach would also enable assessment of the contribution of reduced herbicide uptake to NA activity.

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